Aspirin Resistance

Overestimation of Platelet Aspirin Resistance Detection by Thrombelastograph Platelet Mapping and Validation by Conventional Aggregometry Using Arachidonic Acid Stimulation

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OBJECTIVES This study sought to determine the prevalence of platelet aspirin resistance using methods that directly indicate the degree of platelet cyclooxygenase inhibition.

BACKGROUND Aspirin resistance in platelets may be overestimated by nonspecific laboratory measurements that do not isolate cyclooxygenase activity.

METHODS Arachidonic acid (AA)-induced light-transmittance platelet aggregation (LTA) and thrombelastography (TEG) platelet mapping were performed on the blood of healthy subjects (n = 6) before and 24 h after receiving 325 mg aspirin, and on 223 patients reporting compliance with long-term daily aspirin treatment (n = 203 undergoing percutaneous intervention [PCI] and n = 20 with a history of stent thrombosis). Aspirin resistance was defined as >20% aggregation by LTA or >50% aggregation by TEG.

RESULTS In healthy subjects, AA-induced aggregation by LTA was 82 ± 10% before and 2 ± 1% at 24 h after aspirin (p < 0.001), and aggregation by TEG was 86 ± 14% before and 5 ± 7% at 24 h after aspirin (p < 0.001). In compliant patients, AA-induced aggregation by LTA was 3 ± 2% before PCI and 3 ± 2% after PCI (p = NS), and aggregation by TEG was 5 ± 9% before PCI and 6 ± 14% after PCI (p = NS). Seven PCI patients were noncompliant, and all were aspirin sensitive after in-hospital aspirin treatment. Among 223 patients, only one patient (~0.4%) was resistant to aspirin treatment.

CONCLUSIONS Platelet aspirin resistance assessed by methods that directly indicate inhibition of cyclooxygenase is rare in compliant patients with coronary artery disease. (J Am Coll Cardiol 2005; 46:1705–9) © 2005 by the American College of Cardiology Foundation

The occurrence of ischemic events in patients treated with aspirin has been attributed by some investigators to drug resistance (1–5). Aspirin “resistance” has been assessed by various laboratory methods that assess platelet function after stimulation by agonists such as cationic propyl gallate, arachidonic acid (AA), adenosine diphosphate (ADP), epinephrine, and collagen. Initially, using light-transmittance aggregometry (LTA) with both AA and ADP as agonists, Gum et al. (3) reported a 5% prevalence of aspirin resistance in patients with stable coronary artery disease. With the Ultegra point-of-care Rapid Platelet Function Assay-ASA ( Accumetrics Inc., San Diego, California), in which cationic propyl gallate is used as an agonist, the prevalence of aspirin resistance was 19% to 23% (4,5). Using the PFA-100 (Dade-Behring Inc., West Sacramento, California) point-of-service assay, which uses collagen and epinephrine as the agonists, the prevalence of aspirin resistance was 35% in post-myocardial infarction patients (6). The Working Group on Aspirin Resistance, International Society on Thrombosis and Haemostasis, has written a position statement that summarizes the limitations of the current methods of assessing aspirin responsiveness (7). Because the target of aspirin therapy is inhibition of cyclooxygenase-1 (COX-1), methods that directly indicate the activity of this enzyme would best assess whether aspirin resistance is present in a given patient (8). However, there is no uniformity in either the laboratory methodologies to detect aspirin resistance or the criteria to define aspirin resistance. Moreover, numerous methods that are reported to determine aspirin resistance are strongly affected by pathways of platelet activation other than COX-1. This fact has been proposed as an explanation for the high prevalence of resistance reported in some studies (7–10).

We hypothesized that the prevalence of platelet aspirin resistance is rare when assessed by methods that directly measure the inhibition of platelet COX-1 activity by aspirin. COX-1 converts AA into prostaglandin G2/ prostaglandin H2, which then is converted to thromboxane A2 by thromboxane synthase in platelets. Therefore, we conducted a prospective investigation assessing the prevalence of aspirin resistance using methods that employ AA as the agonist. These studies were conducted in platelet-rich plasma (PRP) and also whole blood to...
exclude the influence of leukocytes and erythrocytes on platelet aggregation (11,12).

**METHODS**

**Patients.** The study was approved by the Investigational Review Board of the Sinai Hospital of Baltimore. Two hundred three consecutive patients undergoing coronary stenting were prospectively studied. Twenty patients with a history of stent thrombosis (SAT) were identified by review of medical records and were contacted for the study. All patients reported compliance with long-term aspirin therapy. All patients undergoing stenting received 325 mg aspirin on the day of the procedure and daily thereafter. All patients who suffered SAT were on a maintenance dose of 75 mg/day clopidogrel at the time of the blood draw. Seventy percent of the patients who underwent percutaneous coronary intervention (PCI) were not on a maintenance dose of clopidogrel before the procedure and received a 300- or 600-mg clopidogrel loading dose during the procedure followed by a 75-mg/day maintenance dose thereafter. Thirty percent of the PCI patients were on a 75-mg daily clopidogrel maintenance dose before the procedure and did not receive a further loading dose.

**Control subjects.** Six healthy subjects were studied before and 24 h after receiving 325 mg aspirin.

**Blood samples.** Blood was collected in a blood collection tube containing 3.8% trisodium citrate for LTA and containing 40 USP lithium heparin tube (United States Pharmacopeia, Rockville, Maryland) for thrombelastography (TEG), filled to capacity, and then inverted three to five times for gentle mixing. Blood samples were obtained before clopidogrel loading and heparin and glycoprotein (GP) IIb/IIIa receptor inhibitor administration (baseline), and at 24 h after the procedure in those patients not receiving GP IIb/IIIa inhibitors. In patients receiving GP IIb/IIIa inhibitors, blood samples were evaluated at five to seven days after the procedure. All patients with a history of subacute thrombosis were confirmed to be on aspirin treat-

**platelet function studies.** LTA. The blood-citrate mixture was centrifuged at 120 g for five minutes, and PRP was recovered. The blood-citrate mixture was subjected to further centrifugation at 850 g for five minutes to recover platelet-poor plasma (PPP). Aggregation was assessed using a Chronolog Lumi-aggregometer (model 490, Chronolog, Haverton, Pennsylvania) with the Aggrolink software package as previously described (13). We assessed the aggregation effect of 1 mmol/l and 2 mmol/l AA in the first 40 patients and found no differences between the two AA concentrations (data not shown). Therefore, platelets were stimulated with 1 mmol/l AA in the remaining PRP samples and platelet aggregation was expressed as the maximal percent change in light transmittance from baseline, using PPP as a reference.

**HAEMOSCOPE THROMBELASTOGRAPH (TEG) PLATELET MAPPING ASSAY.** The recent U.S. Food and Drug Administration-approved TEG Platelet Mapping assay (Haemoscope Corporation, Niles, Illinois) relies on the measurement of clot strength to enable a quantitative analysis of platelet function. The assay uses heparin as an anticoagulant to eliminate thrombin activity in the sample. Reptilase and factor XIIIa (activator F) are used to generate a cross-linked fibrin clot to isolate the fibrin contribution to the clot strength (14). The contribution of P2Y₁₂ receptor or COX pathways to the clot formation can be measured by the addition of an appropriate agonist, ADP or AA. Therefore, AA is added to activator F to measure the degree of thromboxane A₂-induced platelet aggregation.

Blood was analyzed according to the manufacturer’s instructions. One milliliter of heparinized blood was transferred to a vial containing kaolin and mixed by inversion. Five hundred microliters of the activated blood was then transferred to a vial containing heparinase and mixed to neutralize heparin. The neutralized blood (360 μl) was immediately added to a heparinase-coated cup and assayed in the TEG analyzer to measure the thrombin-induced clot strength (Mₘₐₙₐₜ) Heparinized blood (340 μl) was added to a non-coated cup containing reptilase and activator F to generate a whole-blood fibrin cross-linked clot in the absence of thrombin generation or platelet stimulation (Mₖᵢᵢ₀). A third sample (340 μl) of heparinized blood was added to a non-heparinase-coated cup in the presence of the activator F and AA (1 mmol/l) to generate whole-blood cross-linked clot with platelet activation (Mₘₐₜₙₐₜ). Platelet aggregation in response to AA is calculated using computerized software based on the formula: % aggregation = [(Mₘₐₜₙₐₜ - Mₖᵢᵢ₀) / (Mₘₐₙₐₜ - Mₖᵢᵢ₀)] × 100.

**Definition of aspirin resistance.** In this study we measured platelet response to aspirin therapy by measuring the aggregation in both PRP (LTA) and whole blood (TEG) with 1 mmol/l AA as the agonist. Because whole-blood
aggregation in the TEG assay includes fibrin generation and the interaction of platelets with fibrin, higher values of aggregation are expected compared with data obtained from PRP. Therefore, aspirin resistance is defined as the occurrence of more than 20% platelet aggregation after stimulation by 1 mmol/l AA as measured by LTA or more than 50% platelet aggregation after stimulation by 1 mmol/l AA as measured by TEG after aspirin therapy.

**Statistical analysis.** Comparisons were made within groups between baseline and post-treatment by t tests, and p < 0.05 was considered significant. Based on the normal distribution of data, the mean value ± standard deviation was used. A standard regression analysis was performed to correlate aggregation by conventional LTA with TEG measurements of aggregation. In the present study, we hypothesized that by using light transmittance aggregometry with 1 mmol/l AA as the agonist, the prevalence of aspirin resistance is ~5%. Based on the formula: \( n = \frac{15.4 \times p \times (1 - p)}{w^2} \), in which \( n \) = number of patients required, \( p \) = prevalence of aspirin resistance (5%), and \( w \) is the desired width of 95% confidence interval (± 6%), 200 patients were required to complete the study.

**RESULTS**

**Healthy subjects and patients.** Healthy subjects were 35 ± 5 years old and had no modifiable cardiovascular risk factors. Among the patients undergoing PCI, all procedures performed were non-emergent; 36 patients were admitted with unstable angina, and 11 patients had non–ST-segment elevation myocardial infarction. The remainder of the patients had stable angina. In the patients with a history of SAT, the mean time from the index procedure to SAT was 23 ± 16 days. The average time from the occurrence of SAT to blood drawing for laboratory evaluation was 218 ± 204 days. The patient demographics and procedural characteristics of patients undergoing PCI and patients with SAT are shown in **Table 1.** All patients undergoing PCI and all patients who suffered SAT reported taking aspirin 325 mg daily.

**Pre-treatment aggregation.** **CONTROL SUBJECTS.** The LTA and TEG analyses were completed in all patients (n = 223) and healthy subjects (n = 6). In healthy subjects, AA-induced aggregation by LTA was 82 ± 10% and by TEG was 86 ± 14% before aspirin, and it decreased significantly after aspirin treatment (2 ± 1% by LTA and 5 ± 7% by TEG, respectively [p < 0.001]), indicating the complete inhibition of COX-1 by a 325-mg aspirin dose (Figs. 1 and 2).

**PCI PATIENTS.** In patients undergoing PCI (n = 203), all of whom claimed compliance with aspirin therapy, pre-PCI AA-induced platelet aggregation by LTA and TEG was low (5 ± 14% and 7 ± 17%, respectively). Seven PCI patients had high aggregation by LTA and TEG and admitted non-compliance with aspirin therapy. In these non-compliant patients, aggregation by LTA was 73 ± 21% before PCI and decreased to 2 ± 2% post-PCI after in-hospital treatment with 325 mg aspirin (p < 0.001). Similarly, aggregation by TEG was 72 ± 27% before PCI and 7 ± 13% post-PCI after treatment with 325 mg aspirin (p < 0.001). Thus, all patients in the non-compliant group were found to be aspirin sensitive.

All patients in the compliant group (n = 196) were aspirin sensitive. Pre-PCI and post-PCI aggregation by LTA was 3 ± 2% and 3 ± 2%, respectively (p = NS).

![Figure 1. Scatter plot showing 1 mmol/l arachidonic acid-induced platelet aggregation by light-transmittance aggregometry in individual healthy subjects (n = 6) (before and after aspirin therapy), compliant (n = 196) and non-compliant patients (n = 7) undergoing percutaneous interventions (PCIs) (before and after PCI), and patients with a history of stent thrombosis (SAT).](image-url)
Figure 2. Scatter plot showing 1 mmol/l arachidonic acid-induced platelet aggregation by thrombelastography in individual healthy subjects (n = 6) (before and after aspirin therapy), compliant (n = 196) and non-compliant patients (n = 7) undergoing percutaneous interventions (PCIs) (before and after PCI), and patients with a history of stent thrombosis (SAT).

Aggregation by TEG was 4 ± 9% before PCI and 6 ± 14% after PCI (p = NS).

PATIENTS WITH SAT. In patients with SAT (n = 20), AA-induced platelet aggregation by LTA was 6 ± 12% and by TEG was 9 ± 20%. One patient met the definition of aspirin resistance; AA-induced aggregation was 54% by LTA and 85% by TEG in that patient. Therefore, among 223 patients with coronary artery disease evaluated in our investigation, only one patient (~0.4%), who also had a history of SAT, was resistant to aspirin treatment.

CORRELATION OF AGGREGATION MEASURED BY LTA AND TEG. The PRP aggregation measured by LTA strongly correlated with aggregation measured in whole blood by TEG (r = 0.85, p < 0.001). All of the non-compliant patients had high aggregation by both TEG and LTA (Figs. 1 and 2).

DISCUSSION

The present study suggests that aspirin resistance is rare in compliant patients with coronary artery disease when assessed by methods directly dependent on platelet COX-1. In our study, only one of 223 patients (~0.4%) was resistant to aspirin treatment. The concordant data in whole blood and PRP suggest that other cells do not significantly influence ex vivo platelet responsiveness to aspirin.

The antiplatelet effect of aspirin is based on its irreversible acetylation of cyclooxygenase, specifically the COX-1 isoenzyme in platelets. The COX-1 enzyme catalyzes the conversion of AA to prostaglandin H2 and subsequent synthesis of thromboxane A2, a potent platelet agonist. Thus the ideal laboratory evaluation of the antiplatelet effect of aspirin should include the functional response of platelets to AA or measurement of the levels of thromboxane B2, a stable metabolite of thromboxane A2.

The earlier studies of Gum et al. (3) used >20% AA-induced platelet aggregation and >70% ADP-induced aggregation as criteria for defining aspirin resistance. The concentration of AA used by Gum et al. (3) was 1.6 mmol/l, whereas we analyzed AA-induced aggregation at 1.0 mmol/l. The differences in the estimate of resistance based on AA-induced aggregation between the two studies may be in part caused by the difference in the AA concentration used in two studies. However, we compared the effect of 1 mmol/l AA and 2 mmol/l AA on platelet aggregation in the first 40 patients and found no difference in platelet aggregation. Therefore, on the remaining patients we used 1 mmol/l AA. Finally, the patient population studied, the dose of aspirin used, and also the strict in-hospital compliance measures used in our study may have affected the results.

Since the original study by Gum et al. (3), other investigators have used various methods such as the bleeding time and cationic propyl gallate-, ADP-, epinephrine-, and collagen-induced aggregation to assess aspirin responsiveness (1–6). The latter methods affect platelet aggregation by pathways that include but are not specific to COX-1 (7–8). Moreover, the definition of aspirin resistance also varied depending on the assay. Recently, aspirin resistance has been reported by using point-of-service assays. The PFA-100 assay is partially dependent on epinephrine-induced aggregation and thus does not solely isolate COX activity (6). The Ultegra assay estimated aspirin resistance by using cationic propyl gallate to indirectly influence AA metabolism (4,5). Thus, stimulation with a known concentration of AA, a direct substrate of the COX-1 enzyme, was not conducted in the latter studies.

In the present study, we evaluated the platelet response to aspirin in PRP and in whole blood. Moreover, both methods strongly correlated in measuring AA-induced platelet aggregation. Finally, we evaluated the efficacy of 325-mg aspirin therapy in patients and healthy subjects. Among the 223 patients enrolled in the study, ~3% were found non-compliant with aspirin therapy and were therefore pseudo non-responders. This finding is concordant with previous reports (15,16). In-hospital treatment of these non-compliant patients with 325 mg aspirin showed that they were indeed sensitive to aspirin therapy. Only one patient who suffered an SAT was found to be resistant to aspirin therapy as measured by both LTA and TEG. The latter finding suggests that aspirin non-responsiveness may be a risk factor for ischemic events after stenting, as has been reported by other investigators (1–6).

The rare occurrence of aspirin resistance in the present study strongly suggests that the occurrence of aspirin resistance in published reports is overestimated. As can be seen with the non-compliant subgroup of patients in our study, outpatient treatment and lack of compliance are important considerations in determining the prevalence of aspirin resistance. In addition, use of COX-1-specific laboratory analyses such as platelet aggregation in response to AA may show the actual prevalence of aspirin resistance in study populations. This hypothesis is strongly supported by a very recent study of Schwartz et al. (17), in which the prevalence of the aspirin resistance was studied in 190 patients with a
history of myocardial infarction using AA-stimulated light transmittance aggregometry. These investigators found that only one patient was resistant to aspirin therapy. Therefore, the application of specific methods directly indicating COX-1 activity in platelets is recommended to indicate the efficacy of aspirin therapy (7,8).

**Study limitations.** This study is not powered to show the relationship of aspirin resistance and demographic variables. However, the study population included both patients admitted to the hospital for PCI and patients who had suffered SAT despite dual antiplatelet therapy. We did not measure thromboxane metabolite levels or expression of activation-dependent surface molecules, which may have further strengthened our conclusion that platelet aspirin resistance is rare.

**Conclusions.** The result of the present study indicates that aspirin resistance may be overestimated by previous reports using nonspecific laboratory measurements that do not isolate aspirin's primary target, platelet COX-1. In the current study, the target of aspirin therapy was assessed by techniques using whole blood and PRP, and aspirin resistance was nearly absent. Measurements of platelet response to aspirin using more specific methods targeting COX-1 enzyme activity and also a standardized definition of aspirin resistance are warranted to ultimately link aspirin-dependent tests to clinical outcomes in patients with coronary artery disease.

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**REFERENCES**